RNA Extraction

VERSION 1.8 (4/2020)

TR Ex Transcriptional Regulation & Expression Facility

Sample Lysis/Homogenization in Trizol

Equipment and Supplies

- ☐ Trizol (Thermo 15596018 or 15596026) for adherent cells, cell pellets, or tissue samples; or Trizol LS (Thermo 10296010 or 10296028) for suspension cells.
- ☐ RNAse-free 1.5mL or 2mL tubes (compatible with phenol)
- ☐ Recommended for tissue homogenization: Bead mill (e.g. Mini Bead Mill, VWR 10158-558) and pre-filled tubes:
  - ☐ Animal tissue: Hard Tissue Homogenizing Mix, 2.8mm Ceramic beads, 2ml tubes (VWR 10158-612 or Omni International 19-628)
  - ☐ Bacteria: 0.1mm glass bead tubes, 2ml (Omni International 19-621)
  - ☐ Plants: stainless steel beads may work best to crush plant tissue (disposable or reusable)

Trizol Volume

The standard Trizol extraction protocol is designed for 1 mL Trizol. However, the amount of Trizol required for sample lysis depends on the sample type and size. For samples requiring > 1mL Trizol, divide the sample into 1mL aliquots after cell lysis/homogenization and prior to RNA extraction.

Sample Type	Sample Amount	Trizol Amount
Tissue	up to 100 mg (3 mm cube ~ 30-35mg)	1 mL
	per 50-100 mg (5 mm cube ~ 150-175 mg)	1 mL
Adherent cells (monolayer) *scale by surface area*	35 mm dish or 6-well plate (~10cm^2)	1 mL
	60 mm dish or T25 flask (~30cm^2)	3 mL
	100 mm dish or T75 flask (~80 cm^2)	8 mL
Suspension cells	**per** 250 µL (max 5-10 x 10^6 cells)	750 µL (Trizol LS)
Cell pellet	Recommended: resuspend in <100ul vol	1 mL per 10^6 cells
FACS	*See Suspension cells or Cell pellet*	

General Tips

- Always shake Trizol or Trizol LS before use, as contents may stratify during storage.
- Trizol lysates are stable at room temperature for short periods. After homogenization/lysis, gently shaking samples for 5-10 minutes can help ensure lysis is complete.
- Trizol lysates are stable at -80°C for long periods – more stable than storing frozen cells or tissue. Tissue lysates can be frozen in bead mill tubes following homogenization. A freeze/thaw step may help complete lysis and improve RNA yield, and offers a safe-stop before RNA Extraction.
- **Do not** vortex Trizol lysates as it can sheer RNA. (Vortexing is a different mechanical action than bead mill homogenization.)

Lysis Protocol for Different Sample Types

- **Adherent cultured cells:** Lyse directly on tissue culture plates/flasks with appropriate volume Trizol. Do not wash with PBS, just remove all media and add Trizol. Shake or swirl gently until sample is not viscous or sticky before transferring to a 1.5mL tube (can be several minutes).
- **Cells in Suspension:** Use Trizol LS (3:1 Trizol LS:cell volume ratio). Mix immediately by inversion/shaking (process one sample at a time).
- **Cells from FACS:**

  Option 1 – sort directly into Trizol (if added volume is small) or Trizol LS (if added volume is large; adjust final volume for 3:1 ratio). During long sorts, pause and mix sample by inversion.

  Option 2 - Sort up to 1M cells into chilled 100ul PBS containing 20% FBS and 2 mM EDTA (or other buffer that will keep cells happy); hold on ice. After sort, add 3 volumes Trizol LS and immediately mix well by inversion/shaking (process one sample at a time).

  Option 3 - Sort up to 1M cells into chilled 100ul PBS containing 20% FBS and 2 mM EDTA (or other buffer that will keep cells happy); hold on ice. After sort, centrifuge at ≤ 1,000 x g for 5 minutes to pellet cells. Aspirate most of the supernatant with a pipette, leaving less than 100ul volume. Gently flick tube to re-suspend the cell pellet in the residual supernatant. Add 1 mL Trizol and immediately mix well by inversion/shaking (process one sample at a time).
- **Cell Pellets:** Avoid lysing dry cell pellets when possible – soften or re-suspend the pellet in PBS or media and use Trizol LS (3:1 Trizol LS:cell volume ratio), or >9x volumes of Trizol. Treat frozen cell pellets like tissue samples and homogenize immediately, ideally with a bead mill (beads optional). C*ell lysates are more stable for long term storage than cell pellets!*
- **Flash-frozen Tissue:** Rapidly homogenize in Trizol with a bead mill/tissue homogenizer. Alternatively, tissue may be ground to a powder on dry ice or over liquid nitrogen before adding Trizol. *Keep tissue frozen at all times until Trizol is added; process one sample at a time.*
- **Fresh Tissue:** Immediately homogenize in Trizol with a bead mill/tissue homogenizer.
- **Tissue in RNAlater:** If frozen, allow to thaw at room temperature (this is OK, RNA is stable!) and try to resuspend any salt crystals. Remove as much RNAlater as possible from the tissue before homogenization (e.g. blot on kim-wipe) to reduce salt carryover.

Example setup for tissue homogenization*: Prepare labelled bead mill tubes with 1mL Trizol on ice. For flash-frozen samples, prepare a shallow Styrofoam box with dry ice + samples. [Optional: for large frozen samples, chill a metal block and disposable weigh boat on the dry ice. Tip samples into the weigh boat to cut with a single-use knife or razor blade; do not allow to thaw.] Quickly transfer a small piece (~3mm diameter) to a pre-chilled bead mill tube containing 1ml Trizol. Homogenize immediately for 30sec; chill on ice for 30-60sec. Repeat bead mill homogenization as needed until tissue is adequately disrupted, chilling on ice between runs.*

Isolation of total RNA with Trizol

Reagents and Supplies

- ☐ Chloroform (Molecular Biology grade), +/- isoamyl alcohol (e.g. Sigma C2432-500ml)
- ☐ 2ml Phaselock Gel Heavy tubes (VWR 01847-802)
- ☐ GlycoBlue (Thermo AM9515) – recommended
- ☐ Isopropyl alcohol (isopropanol, Molecular Biology grade)
- ☐ Chilled 75% ethanol (made fresh with RNAse-free water)
- ☐ RNAse-free 1.5mL tubes
- ☐ RNAse-free water
- ☐ Aerosol barrier pipette tips

Equipment

- ☐ Refrigerated centrifuge capable of up to 16,000 x g.

Tips for Handling RNA

- Work in a clean area: RNAse-Zap (Thermo AM 9780) can help reduce RNAses. Avoid areas where purified RNAses are used (such as for plasmid DNA isolation). Do not sneeze or cough onto the work area.
- Always wear gloves and use aerosol barrier tips when working with RNA samples. Protect the samples from yourself and your pipets!
- Do not vortex Trizol lysates or RNA samples to avoid shearing.
- After extraction, keep RNA samples on ice at all times.

Initial Sample Preparation

This protocol is designed for samples lysed 1mL of Trizol in a 1.5 or 2mL tube.

- If needed, start with TREx Protocol: Sample Lysis in Trizol.
- If the sample is frozen, thaw at room temperature, with shaking if possible.
- If the sample is in < 1mL Trizol, bring the volume up to 1mL by adding more Trizol.
- If samples are in a bead mill tube, transfer the sample to a clean RNAse-free 1.5 ml tube.
- If the only goal is to isolate RNA and there is particulate matter in the lysate, you may pellet particulates (and gDNA) by centrifugation for 10min @ 12,000xg at 4°C. Transfer the Trizol supernatant to a clean RNAse-free 1.5 mL tube before starting.

Extraction Protocol: Steps 1, 5, and 10 should be done in a laminar flow (chemical) hood.

1. Add 0.2mL chloroform to 1mL of Trizol lysate (containing lysed sample).
2. Shake hard by hand for at least 15 seconds - do NOT vortex. The sample should be light pink.
3. Centrifuge for 15min @ 16,000 x g at 4°C.
4. During spin (or in advance), prepare Phaselock gel (PLG) 2mL tubes:
   a. Label PLG tubes and pre-spin for 30 seconds @12,000 x g at room temperature.
   b. Add 600ul chloroform to the pre-spun PLG tubes and hold at room temperature (*do not chill!*).
5. As soon as 15min spin (step 3) finishes: gently remove samples from the centrifuge; carefully pipet to remove the aqueous phase (top layer) and transfer to a PLG tube containing chloroform.
   a. **Follow the meniscus of the aqueous layer down as you aspirate. Take care to avoid touching the interface between the aqueous and lower solvent layer!** You can remove the sample in one step (P1000) or two (majority with P1000, then switch to P200 for the last ~20%). *Less (sample) is more (pure): don't get greedy!*
   b. Optional: Save interface and/or organic phase for gDNA and/or protein isolation.
6. Shake the PLG tube to mix well - do NOT vortex.
7. Centrifuge 5 min @ 12,000 x g at 4°C.
8. During spin, prepare tubes for precipitation: Label a new RNAse-free 1.5-2mL tube for each sample and add 0.5mL isopropanol.
9. Optional: when spin (step 7) is complete, add 1-2uL GlycoBlue to the top layer of PLG tube. *Glycogen carrier can improve RNA recovery; for low yield samples (e.g. <1M cells) add 2uL.*
10. Transfer the aqueous phase (top layer) from the PLG tube to the new tube containing isopropanol. The phase lock gel should form a barrier between the aqueous and organic phases.
    a. **Try to remove the top aqueous phase without touching or disturbing the phase-lock gel.** If the tip touches the phase-lock gel, do not insert the tip into the isopropanol when you transfer the sample - instead dispense the sample above the level of the isopropanol to avoid transferring the phase-lock gel to the isopropanol.
    b. If the phase-lock gel sits above the aqueous phase, and does not 'lock' between the aqueous and chloroform layers, the sample may be too salty. Add 50ul RNAse-free water to the phase-lock tube, shake, and spin again.
11. Mix well by inverting the precipitation tubes – do NOT vortex.
12. Incubate for at least 10 minutes, max 30 minutes at room temperature (shaking recommended). *For low yield samples, incubate as low as 4°C for up to 1 hour with shaking (eg 700rpm in Thermomixer). Lower incubation temperatures may increase salt carryover. Freezing samples at the first precipitation step will likely cause salts to precipitate and is not recommended.*
13. Centrifuge at least 10 minutes @16,000 x g at 4°C. Spin the tubes in a consistent orientation (e.g. hinge up) to make the RNA pellet easier to find. *Longer spin times may improve yield; centrifuge low yield samples for 30 minutes.*

14. Pour off supernatant into a new 1.5mL waste tube – watch for slippery pellets. Pellet will appear blue if GlycoBlue was added. *Pouring into a new tube instead of into a waste container allows recovery of the pellet if needed (repeat centrifugation step).*
    For full removal of residual salts, pulse-spin the tube after pouring off the supernatant and remove remaining volume with fine tip (e.g. P20) – at this step and each wash step below.
15. Wash pellet with 1mL cold 75% ethanol. Invert the tube several times to mix – do NOT vortex. *Mixing is important to wash the pellet as it helps remove residual salt. During this process the pellet with easily come loose. This is OK, as it helps to thoroughly wash the pellet.*
16. Centrifuge for 5 min @ 12,000-16,000 x g at 4°C (*low yield samples: spin 10 minutes*).
17. Pour off or transfer supernatant to a new waste tube – watch for slippery RNA pellets.
18. Wash again with 1mL cold 75% ethanol. Invert the tube gently several times to mix – do NOT vortex. *Optional: store sample as RNA pellet in 75% ethanol at ≤ -20°C overnight.*
19. Centrifuge for 5 min @ 12,000-16,000 x g at 4°C (*low yield samples: spin 10 minutes*).
20. Pour off or remove supernatant to a new waste tube – watch for slippery RNA pellets.
21. Optional: If RNA chemical purity is a concern (e.g. for very low yield samples, or when using RNA in enzymatic reactions that are sensitive to salt – such as small RNA library prep), a third ethanol wash may help further reduce salt carryover. Most samples do not require a third ethanol wash.
22. Recommended: pulse-spin the tube after pouring off the final wash and remove the residual ethanol wash volume with a fine tip (eg P20).
23. Air-dry the pellet in a laminar flow hood for 5-10min (or longer on a bench). You can also dry briefly in a speed-vac to evaporate ethanol, but do not over-dry the RNA pellet.
24. Re-suspend RNA sample in RNase-free water, and **keep on ice from this step forward**.

Recommended volume:	low yield samples (<1M cells)	20 ul
	high yield samples	30-50 ul (or more)

25. Quantify high yield samples with a Nanodrop or low yield samples (<25 ng/ul) with a HS RNA Qubit assay. Ultra-low yield samples (<5 ng/ul) may require several microliters to detect with a Qubit assay, which can consume a considerable portion of the sample and may not be worth it.
26. Optional: prepare a 3-5ul aliquot for Fragment Analyzer QC. Dilute as needed to the target concentration range with RNAse-free water.

Sensitivity Mode	Sample conc.	Notes
Standard	30-200ng/ul	*For samples <30ng/ul dilute to High Sensitivity*
High	3-5ng/ul	*Can go as low as 1ng/ul but will have low signal*
Femto	< 250pg/ul	*Ultra-sensitive; $15/sample*

27. Optional: prepare additional RNA aliquots for downstream applications, if desired. This can help avoid repeat freeze-thaws of the primary RNA sample.
28. Store RNA samples at -80°C.
29. Handle Trizol, chloroform, and ethanol waste per EHS guidelines.